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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/82, A01H 5/10</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/27068</b> <b>(43) International Publication Date:</b> 12 October 1995 (12.10.95)
<b>(21) International Application Number:</b> PCT/US95/03784 <b>(22) International Filing Date:</b> 24 March 1995 (24.03.95) <b>(30) Priority Data:</b> 08/222,148 4 April 1994 (04.04.94) US <b>(71) Applicant:</b> PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US). <b>(72) Inventors:</b> BEACH, Larry; 3939 Maquoketa Drive, Des Moines, IA 50311 (US). ORMAN, Bruce; 502 S.E. 6th Street, Ankeny, IA 50021 (US). TOWNSEND, Jeffrey; 2988 N.W. 132 Ct., West Des Moines, IA 50365 (US). THOMAS, Laurie; 2447 Walnut Avenue, Venice, CA 90291 (US). <b>(74) Agents:</b> SIMON, Soma, G. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> REDUCTION OF ENDOGENOUS SEED PROTEIN LEVELS IN PLANTS  <b>(57) Abstract</b>  A plant seed and method of producing the same, genetically modified to express a preselected protein, the expression of which reduces the level of an endogenous protein in the seed. In particular, expression of a sulfur-rich, seed storage protein reduces the level of protease inhibitors in seeds.		

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## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_ because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>2</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_ because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheets

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:  
1-21, 23-24, 26-41 and 44-46 to the extent they read on the elected species.
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☒ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT(Continued)

- Y,P      Biological Abstracts Volume 87(4) Issued 15 February 1989 SWEDER ET AL. "Purification and characterization of proteins that bind to yeast ARSs" See ref. 37597. 1-21,23-24,26-41 and 44-46
- Y      Biological Abstracts Volume 85(9) Issued 01 May 1988 HAUBER ET AL. "Mutational analysis of the trans-activation responsive region of the human immunodeficiency virus type long terminal repeat" See reference 89626. 1-21,23-24,26-41 and 44-46
- Y      Life Sciences Collection Accession Number 82001774133 Issued 1988 HENDERSON "Isolation and characterization of a novel protein (X-ORF) product from ISIV and HIV-2". See abstract. 1-21,23-24,26-41 and 44-46
- Y      Life Sciences Collection Accession Number 82001901798 Issued 1988 "Purification of the human immunodeficiency virus type 1 enhancer and TAR binding proteins EBP-1 and UBP-1". See abstract. 1-21,23-24,26-41 and 44-46

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/00024

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>1</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00; C12Q 1/08  
U.S. CL.: 435/6; 435/69.1

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System

Classification Symbols

U.S. 435/69/1, 172.3, 6, 240.1  
935/8, 11, 16, 19

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

DNA databases searched. Palago "biotech" database  
Searched: DNA binding protein, affinity, oligonucleotide

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>15</sup>
Y,P	<u>Biochemistry</u> , Volume 28, No. 23, Issued 1989, HUGHES ET AL., "Purification and characterization of a protein from Hela cells that binds with high affinity to the estrogen response element, GGTCAGCGTGACC" See pages 9137-9142.	1-21,23-24,26-41 and 44-46
Y	<u>Proceedings of the National Academy of Sciences USA</u> Volume 85 issued December 1988 GAYNOR ET AL. "REPEATED B motifs in the human immunodeficiency virus type I long terminal repeat enhancer region do not exhibit cooperative factor binding" See pages 9406-9410.	1-21,23-24,26-41 and 44-46
Y,P	<u>Journal of Virology</u> Volume 63(6) Issued June 1989 HARRICH ET AL. "Role of SPI-binding domains in the in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat" See pages 2585-2591.	1-21,23-24,26-41 and 44-46

<sup>6</sup> Special categories of cited documents: <sup>15</sup>

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## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>1</sup>

18 JUNE 1990

International Searching Authority <sup>1</sup>

ISA/US

Date of Mailing of this International Search Report <sup>1</sup>

16 JUL 1990

Signature of Authorized Officer <sup>10</sup>

MICHELLE MARKS

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/03784

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9214822	03-09-92	AU-A- 1353892 BG-A- 98042 CZ-A- 9301679 EP-A- 0571500 JP-T- 6505387	15-09-92 30-06-94 16-03-94 01-12-93 23-06-94
EP-A-0318341	31-05-89	AU-A- 2811889 WO-A- 8903887 EP-A- 0319353 JP-T- 2501802 AU-B- 634987 AU-A- 4495189 WO-A- 9004032 JP-T- 3502644	23-05-89 05-05-89 07-06-89 21-06-90 11-03-93 01-05-90 19-04-90 20-06-91
WO-A-9113993	19-09-91	AU-A- 7583691 EP-A- 0519011	10-10-91 23-12-92
WO-A-9303160	18-02-93	AU-A- 2441292 CA-A- 2114788 EP-A- 0598806 JP-T- 7502163	02-03-93 18-02-93 01-06-94 09-03-95
WO-A-9318643	30-09-93	AU-B- 1423992 EP-A- 0591530	21-10-93 13-04-94

## INTERNATIONAL SEARCH REPORT

Intern

Application No

PCT/US 95/03784

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PLANT PHYSIOLOGY, vol. 104, February 1994 pages 497-503, NAITO, S., ET AL. 'EXPRESSION OF A SOYBEAN (GLYCINE MAX.[L.]MERR.) SEED STORAGE PROTEIN GENE IN TRANSGENIC ARABIDOPSIS THALIANA AND ITS RESPONSE TO NUTRITIONAL STRESS AND TO ABSCISIC ACID MUTATIONS' cited in the application see the whole document ---	1
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, September 1993 WASHINGTON US, pages 8273-8276, OROZCO-CARDENAS, M., ET AL. 'EXPRESSION OF AN ANTISENSE PROSYSTEMIN GENE IN TOMATO PLANTS REDUCES RESISTANCE TOWARD MANDUCA SEXTA LARVAE' see page 8276, left column ---	1,7
A	WO-A-93 18643 (RICE BREEDING RESEARCH LAB) 30 September 1993 & EP-A-0 591 530 (RICE BREEDING RESEARCH LAB) 13 April 1994 see the whole document -----	1-18



# INTERNATIONAL SEARCH REPORT

Original Application No.

PCT/US 95/03784

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. CELL. BIOCHEM. SUPPL. 18A, 1994 page 79 FALCO, S.C., ET AL. 'TRANSGENIC CROPS WITH IMPROVED AMINO ACID COMPOSITION ' see abstract X1-017 & KEYSTONE SYMPOSIUM, IMPROVED CROP AND PLANT PRODUCTS THROUGH BIOTECHNOLOGY, HELD JAN. 9-16, 1994, ---	1,5
X	PLANT MOLECULAR BIOLOGY, vol. 18, 1992 pages 235-245, ALTENBACH, S.B., ET AL. 'ACCUMULATION OF A BRAZIL NUT ALBUMIN IN SEEDS OF TRANSGENIC CANOLA RESULTS IN ENHANCED LEVELS OF SEED PROTEIN METHIONINE' see the whole document ---	1
X	CURRENT TOP. PLANT PHYSIOL., vol. 7, 1992 pages 229-238, BEACH, L. R., ET AL. 'ENHANCING NUTRITIONAL VALUE OF SEED CROPS' see page 233, paragraph 1 - paragraph 3 ---	1
X	EP-A-0 318 341 (PLANT GENETIC SYSTEMS NV) 31 May 1989 see the whole document ---	1
X	WO-A-91 13993 (UPJOHN CO) 19 September 1991 see the whole document ---	1
X	WO-A-93 03160 (DU PONT) 18 February 1993 see the whole document ---	1
A	PLANT MOLECULAR BIOLOGY, vol. 13, 1989 pages 513-522, ALTENBACH, S.B., ET AL. 'ENHANCEMENT OF THE METHIONINE CONTENT OF SEED PROTEINS BY THE EXPRESSION OF A CHOMERIC GENE ENCODING A METHIONINE-RICH PROTEIN IN TRANSGENIC PLANTS' see page 516, right column - page 517, left column --- -/--	1-3

# INTERNATIONAL SEARCH REPORT

Inter Application No  
PC/US 95/03784

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/82 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-92 14822 (DU PONT) 3 September 1992 see example 11 ---	1,5
X	J. CELL. BIOCHEM. SUPPL. 18A, 1994 page 107 RICE, J.A., ET AL. 'EXPRESSION OF SYNTHETIC HIGH LYSINE SEED STROAGE PROTEINS CAN SIGNIFICANTLY INCREASE THE ACCUMULATED LEVELS OF LYSINE IN MATURE SEEDS OF TRANSGENIC CROP PLANTS' see abstract X1-329 & KEYSTONE SYMPOSIUM, IMPROVED CROP AND PLANT PRODUCTS THROUGH BIOTECHNOLOGY, HELD JAN 9-16, 1994, --- -/--	1,5

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 July 1995

Date of mailing of the international search report

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Name and mailing address of the ISA

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Authorized officer

Maddox, A

## REDUCTION OF ENDOGENOUS SEED PROTEIN LEVELS IN PLANTS

### Field of the invention

- 5           The present invention relates to the reduction of endogenous seed protein levels in plants.

### Background of the invention

- 10           The seeds of land plants contain large quantities of storage, or reserve, proteins which are synthesized during the development of the seeds. During germination and early seedling growth, these reserves are hydrolyzed to produce metabolic intermediates for use by the growing seedling. In harvested seeds, storage proteins represent an available package of  
15           condensed food and enzymes. The food value of these seeds furthermore, could be increased by altering the composition of the reserve proteins to decrease the amount of undesirable proteins in the seeds.

- It is known that mineral nutrition can have an influence on the composition of seed proteins. Chandler, *et al.* (1983) *Plant Physiol.* 71, 47-  
20           54; and Randall, *et al.* (1979) *Aust. J. Plant Physiol.* 6, 11-24. Because amino acids in plants are the direct products of specific biosynthetic pathways and are required for the formation of polypeptides or plant proteins, the supply of certain minerals can alter the amount of particular amino acids available for polypeptide synthesis and, consequently, the relative  
25           proportions of proteins in the seed. Gengenbach, *et al.*, *Genetic Manipulation of Corn Kernel Amino Acid Composition* (June 20-21, 1990) Corn Utilization Conference II Proceedings, Nat'l Corn Growers Association; Chandler, *et al.* (1984) 75, 651-657.

- Recombinant DNA and gene transfer technologies have been applied  
30           to alter enzyme activity catalyzing key steps in the amino acid biosynthetic pathway. Naito, *et al.*, (1994) *Plant Physiol.* 104, 497-503; Glassman, U.S. Patent No. 5,258,300; Falco, S.C. (1993) PCT Pat nt Appl. WO 93/19190;

Galili, *et al.* (1992) Eur. Patent Appl. EP No.485970. However, modification of the amino acid levels in seeds is not always correlated with changes in the level of proteins that incorporate those amino acids. Burrow, *et al.* (1993) *Mol. Gen. Genet.* 241, 431-439.

5        Gene modification techniques are also being used to increase or decrease the expression of endogenous proteins. However, these techniques have concentrated on direct modification of the DNA encoding the protein, or the introduction of several copies of sequences homologous to the DNA (Jorgensen, U.S. Patents No. 5,034,323 and No. 5,283,184), or  
10       introduction of antisense polynucleotides. Inouye, U.S. Patent No. 5,190,931. Until now, transformation technology has not used expression of one desirable protein to target the reduction of expression of an undesirable native protein in plants.

      Some of the seed storage proteins in most, if not all, plants are in a  
15       class called protease inhibitors. These inhibitors are thought to function not only as storage proteins, but as regulators of endogenous proteases, and as proteins that protect plants from insect and pathogen attack. Liener and Kakade (1980) *Protease Inhibitors*. In *Toxic Constituents of Plant Foodstuffs*, 2nd ed.; Linear, I.E., Ed.; Acad. Press: New York, pp 7-71.; Ryan, CA (1990)  
20       *Ann. Rev. Phytopathol.* 28, 425-449.

      The plant protease inhibitors are generally low molecular weight proteins, and share in common the ability to combine with particular animal, and occasionally plant proteases, thereby abolishing the activity of these enzymes. Wilson, K.A. (1988) *CRC Rev. Biotech.*, 8(3), 197-216. Inhibitors  
25       that are active towards the mammalian enzymes trypsin or chymotrypsin have been best studied. This work suggests that active protease inhibitors may be toxic to humans and other animals, adversely affecting the nutritional quality of plant foodstuffs, even though they may be beneficial under other circumstances. Thus, there is a desire to minimize the amount of protease  
30       inhibitors in foods.

      Protease inhibitors are particularly abundant in the legume family and constitute about 6% of the proteins of soybeans. Brandon, U.S. Patent No.

4,959,310. Their antinutritional nature leads to pancreatic hyperplasia, acinar adenoma, and overall growth reduction when raw soybean meal is fed to monogastric animals, such as chicks, rats, and quail. Chernick, et al. (1948); Am. J. Physiol., 155, 33-41; Gumbmann, et al. (1986) p. 33-80, In  
5 *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods*. M. Friedman (ed.), Plenum Press, New York.

Soybean (*Glycine max*) seed proteins are one example of storage proteins that are widely used in human foods such as infant formulas, tofu, soy protein isolates, soy flour, textured soy fibers, and soy sauce. Soybean  
10 protein products serve as an excellent source of low cost, high quality protein for human needs. Soybeans are also widely used as a component of animal feeds. However, they must be properly processed to remove or deactivate protease inhibitors.

Soybean protease inhibitors are categorized into three classes: Kunitz  
15 trypsin inhibitors, Bowman-Birk inhibitors, and glycine-rich soybean trypsin inhibitors (GRSTI). The primary structure of these inhibitors consists partly of sulfur-containing (methionine and cysteine) amino acids. Kollipara, K. P. and Hymowitz, T. (1992) *J. Agr. Food*; 40, 2356-2363.

The major and predominantly expressed form of Kunitz trypsin  
20 inhibitors (KTI) is a 21.5-kDa protein which has an inhibition specificity for trypsin. Bowman-Birk inhibitor (BBI) is a low molecular weight (8000 kDa) protein that inhibits both trypsin and chymotrypsin simultaneously at independent reactive sites. At least ten different isoforms of BBI have been reported. GRSTI are minor inhibitors of trypsin in soybean seed.

25 Various approaches have been taken to reduce the protease inhibitor content and/or activity of soybeans. These include physical (heat) and chemical treatment of soy products, as well as genetic alteration of soybeans through conventional breeding techniques. Liener and Kakade, *supra*.

In any heat treatment, care must be taken because, even though  
30 heating is required to destroy the trypsin inhibitors, improper heating will result in damage to the protein product itself. Furthermore, although the protease inhibitor activity is largely inactivated by denaturation through

conventionally applied heat treatment of soy flour, 10-15% residual activity usually remains. The unusual structure of the BBI is the most likely reason for this residual activity. BBI is strongly cross-linked by disulfide bonds which gives the molecule resistance to heat denaturation. Thus, heat treatment of seed or soy products to reduce inhibitor expression is not completely successful and furthermore, is costly in energy usage.

The solvent-extraction method is another process used to eliminate protease inhibitors from raw soybeans. This chemical extraction, while removing the various inhibiting materials however, results in considerable loss of the oil in the seed, thus reducing its food value. At the same time, the solvent poses problems of cleanup and disposal.

Genetic modification of the soybean plant to develop low inhibitor activity varieties has also been proposed but has inherent limitations. Desirable nutritional value may be lost concomitant with the reduction of the inhibitors, and cross pollination of the genetic variant with another cultivar could result in reexpression of the protease inhibitor gene. Further, altering expression of one inhibitor may not affect the expression of another. As yet, conventional breeding and tissue culture technology has been unable to produce a soybean plant with low levels of protease inhibitors although a need exists for such plants.

Until now, no method existed to reduce the level of all protease inhibitors in plant seeds. In fact, no method existed to purposely use expression of one protein to reduce the level of another protein through limitation of an amino acid source.

However, we have observed that as a result of expression of a particular protein during the development of seeds, other storage proteins can be reduced in quantity. Specifically, we have concentrated on the reduction of antinutritional proteins through the expression of another preselected protein in soybean that contains sulfur amino acids. Our results indicate that undesirable antinutritional proteins such as protease inhibitors may be successfully reduced by expressing other, more desirable proteins

containing amino acids that are common and essential to both the antinutritional protein and the preselected protein.

While not intending to be limited in theory, it is thought that this method of reducing antinutritional proteins works because the pool of the available amino acids common to both proteins is limited during development of the seeds. Therefore, reducing the source of the amino acid through increased synthesis of the preselected protein inhibits synthesis of the antinutritional protein.

Therefore, it is an object of this invention to provide a novel method to eliminate or reduce the content of endogenous proteins in plant seeds.

It is another object of this invention to provide plant seeds that have a reduced content of an endogenous protein as a result of increasing the content of a preselected protein with enhanced nutritional value.

It is another object of this invention to provide plant seeds that require no time-consuming or costly processing to eliminate protease inhibitor activity.

Still another object of this invention is to provide a novel method to eliminate or reduce antinutritional proteins from soybeans while retaining and even increasing the content of nutritional proteins in soybean seeds.

Yet another object of this invention is to reduce endogenous proteins in seeds by reducing the available amino acids required to synthesize the endogenous proteins.

A still further object of the present invention is to produce soybean seeds with reduced or eliminated protease inhibitor content by reducing the available sulfur-containing amino acids required to synthesize the protease inhibitor by expressing a preselected sulfur-containing protein.

A further object of this invention is to provide a transgenic plant that produces a seed having little or no protease inhibitor content.

Summary of the Invention

In accordance with these objectives, the present invention provides a plant seed that is genetically modified, relative to a wild type of the species of the seed, to preferentially express a preselected protein, whereby the content of a second, endogenous protein is diminished in the seed. The preselected protein in the plant seed can be a second endogenous protein which the seed is genetically modified to overexpress or it can be a heterologous protein. Examples of preselected proteins can be, but are not limited to, a methionine-containing protein, a cysteine-containing protein, a lysine-containing protein, a glycine-rich protein, a tryptophan-containing protein, or a tyrosine-containing protein.

The invention is based on the discovery that, during development, when the plant seed has a finite amount of an amino acid required for synthesis of the antinutritional protein, increased expression of another protein requiring the same amino acid, will result in reduction or elimination of the final content of antinutritional protein in the seed, thus eliminating or reducing the need to further process the seed-based foodstuffs to effect a reduction in content.

Therefore, the present invention provides a plant seed with reduced sulfur-containing, antinutritional proteins resulting from the reduction of sulfur-containing amino acids in developing seeds. The sulfur amino acid pools in developing seeds are redistributed by expressing a sulfur-containing protein in the developing seeds. Further, the preselected protein can be a desirable source of the amino acid in question, thus increasing the food value of the seed.

The present invention also provides a method for reducing the level of an endogenous protein in a seed by altering expression of a second protein in the seed that requires the same amino acid as the endogenous protein.



### Detailed Description of the Invention

As used herein, "genetically modified" means a plant cell stably incorporating a nucleic acid construct introduced by transformation methods.

5 The term "wild type" refers to an untransformed plant cell.

The invention also embraces reducing translation of nucleic acid sequences encoding endogenous proteins. "Endogenous" protein refers to the native protein normally found in its natural location in the plant.

10 In addition, the invention comprises the methods of preparing and using the various DNA constructs of the present invention. Plants, seeds, and microorganisms transformed with the nucleic acid sequences described are also embodiments of the invention.

Preferred plants that produce seeds wherein protein content may be improved by this method include, but are not limited to, soybeans, canola, 15 corn, sunflower, wheat, barley, oats, millet, rice, sorghum, and rye. The seeds may be used directly as feed or food, or further processing may occur. In the practice of the present invention, the most preferred plant seed is *Glycine max*, the preferred endogenous protein whose content is diminished is a protease inhibitor, and the preselected protein is a methionine-rich, seed 20 storage protein.

In accordance with this invention, there is provided a simple, rapid, and reliable process for the production of transgenic soybean plants with reduced protease inhibitor activity in the resulting seeds. The method is genotype independent and shows a substantial improvement over previously- 25 used systems because it eliminates or considerably reduces necessary, time-consuming, and costly steps to eliminate protease inhibitor activity from soy food products.

As used herein, "promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of 30 the coding sequence by providing the recognition for RNA polymerase and other factors required for promoter transcription. Preferred promoters are those that allow expression of the preselected protein specifically in seeds to

- avoid any potential deleterious effect in non-seed organs. Examples of seed-specific promoters include, but are not limited to, the promoters of seed storage proteins which express these proteins in seeds in a highly regulated manner. Thompson, *et al.* (1989) *BioEssays* 10, 108-113. Several seed
- 5 specific promoters for expression of proteins in seeds of dicotyledonous plants that will be of particular use include bean  $\beta$ -phaseolin, globulin 1, napin,  $\beta$ -conglycinin, and soybean lectin. For monocotyledonous plants, maize 15 kD zein, 22kD zein,  $\gamma$ -zein, waxy, shrunken 1, and shrunken 2 promoters will be particularly useful to produce expression of peptides.
- 10 Those skilled in the art will recognize other promoters as well that will provide constructs for increased levels of the preselected protein in the plant chosen for transformation.

- In a highly preferred embodiment, the preselected protein is a methionine-rich 2S seed storage protein such as Brazil nut protein (BNP).
- 15 Altenbach, *et al.*, (1987) *Plant Mol. Biol.*, 8, 239-250. A natural or constructed DNA or RNA sequence encoding this protein is introduced into plant cells by any method of transformation that stably incorporates the gene into the plant genome. This can include a variety of vectors, such as viral vectors, episomal vectors, shuttle vectors, Ti plasmid vectors and the like, all in
- 20 accordance with well known procedures. Sun, *et al.*, (1991) *Eur. Patent Appl.* EP No. 295,959. A "vector" is a replicon, such as a plasmid, cosmid, or bacteriophage, to which another DNA segment may be attached so as to bring about replication of the attached segment, or to allow its introduction into a cellular host.

- 25 As used herein with respect to a protein, the term "heterologous" means that the gene or gene fragment encoding the protein is obtained from one or more sources other than the genome of the species of plant within which it is ultimately expressed. The source can be natural, e.g., the gene can be obtained from another source of living matter, such as bacteria, yeast,
- 30 fungi and the like, or a different species of plant. The source can also be synthetic, e.g., the gene or gene fragment can be prepared in vitro by chemical synthesis.

As used herein with respect to a preselected protein, the term "expresses" means that the gene encoding this protein is stably incorporated into the genome of the cells, so that the product encoded by the gene, e.g., a methionine-rich protein such as Brazil nut protein (BNP), is produced within the cells. For example, novel plants resulting from expression of BNP, contain extractable seed BNP levels of 0.5%, and preferably, at least 2%. Furthermore, as a result of BNP expression, the endogenous protein levels are diminished 5%, or preferably at least 50% or more. Those skilled in the art will recognize that the levels of extractable protein necessary to reduce endogenous protein levels may vary since different proteins will contain different levels of the desired amino acid residues.

Levels of an endogenous protein in a plant seed are reduced by the use of nucleic acid sequences inserted into the genome of a plant to cause the expression of a preselected protein, the sequence of which requires a limiting amino acid necessary to construct the primary structure of the endogenous protein. Synthesis of the preselected protein removes the source of the amino acid for synthesis of the endogenous protein, thus inhibiting its synthesis and subsequent presence in the seed. The amount of inhibition of the endogenous protein will depend on the location in the genome and the number of copies of the heterologous gene in the genetically-modified cell. These will affect expression of the preselected protein. Transgenic plants will exhibit a variety of different phenotypic expressions of the preselected protein, and selecting plants with high levels of expression can be readily achieved by skilled artisans in accordance with the present invention.

The properties of the nucleic acid sequences encoding the preselected protein may be varied and the preferred embodiment describes a number of features which may be advantageous but that a person skilled in the art will recognize as not being absolutely essential. These include the selection of a particular construct and vector to introduce the sequence into the cell and produce expression of the protein. A skilled artisan can construct an expression cassette adequate for expression of the preselected protein in the

chosen cellular system with no undue experimentation. The heart of the invention is the level of expression of the preselected protein; therefore, additional copies of the nucleic acid sequence will normally result in increased inhibition of synthesis of the endogenous protein.

5 By way of example, and not limitation, those skilled in the art will readily appreciate that additional proteins may be substituted for the BNP protein as the preselected seed protein. The skilled artisan will recognize that choice of the preselected protein will be based on the amino acid composition of the protein and its ability to accumulate in seeds. This  
10 includes all classes of seed storage proteins; the 2S, 7S, and 11S proteins with or without modification to increase the content of the designated amino acid in the protein. The amino acid can be chosen for its nutritional value to produce a value-added trait to the plant as well as its purpose as a sink to limit availability to the designated endogenous protein. Examples of suitable  
15 sources for protein sequences usable in accordance with the present invention are plants, in particular higher plants. Amino acids desirable for value-added traits as well as a source to limit synthesis of an endogenous protein include, but are not limited to methionine, cysteine, glycine, lysine, tryptophan, and tyrosine.

20 As used herein, "plant" refers to either a whole plant, a plant part, a plant cell, or a group of plant cells. The class of plants which can be used in the method of the invention is generally as broad as the class of seed-bearing higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. The transformation of the  
25 plants in accordance with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. These include but are not limited to particle bombardment, microinjection, electroporation, and *Agrobacterium*-mediated DNA transfer.

Following transformation, regeneration will normally be involved in  
30 obtaining a whole plant from the transformation process. Techniques for regenerating plants from tissue culture, such as transformed protoplasts or callus cell lines, are known in the art. See, e.g., Phillips, *et al.* (1981) *Plant*

*Cell Tissue Organ Culture* 1, 123; Patterson, K.E. and N.P. Everett (1985) *Plant Sci.* 42, 125-132; Wright, *et al.* (1987) *Plant Cell Reports* 6,83-89; Barwale, *et al.* (1986) *Planta* 167,473. The selection of an appropriate method is within the skill of the art.

5        Examples of the practice of the present invention detailed herein relate specifically to soybean plants and expression vectors operable in dicots. Soybean was chosen as a model system for these examples primarily because of the present capability to regenerate soybean plants from transformed individual soybean cells in a manner now known in the art. The  
10        expression vectors utilized herein are demonstrably capable of operation in cells of many dicotyledonous plants both in tissue culture and in whole plants. The invention disclosed herein is thus operable in dicotyledonous species to transform individual plant cells and to achieve full, intact plants in dicot plant species which can be regenerated from transformed plant calli and which  
15        express preselected seed proteins. For those species not presently regenerable, the present invention is fully operable when the techniques for such regeneration become developed.

      In addition, chimeric expression vectors involving seed proteins are also known and have been described in the literature which have been  
20        demonstrated to be operable in cells of monocots, at least in tissue culture. It is reasonable then to expect that these vectors will also be operable in whole monocot plants when the techniques for regenerating these plants are perfected so that any preselected seed protein can be expressed in any monocotyledonous plant seed. The present invention is thus applicable to  
25        monocots as well as to dicots.

      Therefore, practice of this invention can be used to improve crop plants like rice, maize, wheat, and barley with few modifications. An example of such an embodiment would be the introduction of a high lysine derivative of  $\alpha$ -hordothionin into a barley or wheat cell to reduce the purothionin content  
30        of the seed and increase its lysine content.

      Thionins are small antimicrobial proteins present in the endosperm of barley, wheat, and other plant species. Florack, *et al.* (1994) *Plant Mol. Biol.*

24, 83-96. Native  $\alpha$ -hordothionin is rich in arginine and lysine residues, containing five residues (10%) of each. Several derivatives of this protein have been made in which other amino acids were replaced with lysine to produce a compound less toxic to fungi and significantly more enriched with lysine (29% lysine).

Purothionins are also small, lysine-rich proteins in the endosperm of wheat and several other species of *Gramineae*. Wada, K. (1982) *Plant & Cell Physiol.* 23(8), 1357-1361. Purothionins are lethal to brewer's yeast and, as a result, barley or wheat with high levels of these proteins cannot be used for making high quality beers.

However, according to this invention, a high-lysine  $\alpha$ -hordothionin or another genetically-engineered thionin designed for lysine enrichment and reduced toxicity to microorganisms could be used to decrease the levels of purothionins and increase the lysine content of barley, wheat, or other graminaceous plants. The lysine-enriched residue could be sold for feed following the brewing process.

The foregoing is one description of the scope of the invention and a skilled artisan will recognize many other examples of plant improvement to which the invention can be applied.

The present invention can be better understood by reference to the following more detailed example which illustrates its various applications, but is in no way intended to limit the scope thereof.

### Experimental

#### Transformation of *Glycine max* with a Methionine-rich Seed Storage Protein

##### Plant transformation

Soybean (*Glycine max*) seed, Pioneer variety 9341, was surface sterilized by exposure to chlorine gas evolved in a glass bell jar. Gas was produced by adding 3.5 ml hydrochloric acid (34-37% w/w) to 100 ml sodium

hypochlorit (5.25% w/w). Exposure was for 16-20 hours in a container approximately on cubic foot in volume. Surface sterilized seed was stored in petri dishes at room temperature. Seed was germinated by plating on 1/10 strength agar solidified medium according to Gamborg [B5 basal medium with minimal organics, Sigma Chemical Co., cat. no. G5893, 0.32 gm/L; sucrose, 0.2% w/v and 2-[N-morpholino]ethanesulfonic acid (MES), 3.0 mM] without plant growth regulators and culturing at 28°C with a 16 hour day length and cool white fluorescent illumination of approximately 20 mEm2S1. After three or four days, seed could be prepared for cocultivation. The seed coat was removed and the elongating radicle was removed 3-4 mm below the cotyledons. Ten prepared seeds were held in each of several petri dishes.

#### Construction of plasmids

For construction of the plasmid p12GUSBN17, containing one copy of the chimeric methionine-rich protein gene (BNP), we used the plasmid pARC12 (Prosen D.E. and R.B. Simpson (1987) *Biotechnology* 5, 966-971). This is a 29.5 kb plasmid which is part of a binary vector system of *Agrobacterium* and contains the chimeric gene nopaline synthase/neomycin phosphotransferase II as a selectable marker for plant cells. The chimeric gene, CaMV35S/ $\beta$ -glucuronidase, obtained from the plasmid pB1221 (Jefferson, R.A. (1987) *Plant Mol. Bio. Reporter* 5(4), 387-405) was inserted into pARC12, resulting in plasmid p12GUS-15. The plasmid pD3-8-12 (Altenbach, et al. (1989), *Plant Mol. Biol.* 13, 513-522), contains the BNP gene in the vector pTZ19U. The pD3-8-12 plasmid was cleaved with Hind III and inserted into the Hind III site of plasmid p12GUS-15. The resulting plasmid p12GUSBN17 is about 36 kb in size, contains one copy of the BNP gene, and confers resistance to ampicillin and tetracycline to the bacterial host.

For the construction of a plasmid containing four copies of the methionine-rich protein gene, the plasmid pD3-8-12 was used as the starting point. The BNP gene was excised from pD3-8-12 by digestion with Eco R1,

Hind III, and Xmn I. The ends of the fragment were made blunt with the Klenow fragment of DNA polymerase, and a 3 kb fragment containing the chimeric gene was gel-purified. This fragment was ligated to the plasmid pD3-8-12 which had been digested with Sma I and treated with calf intestinal phosphatase. The resulting plasmid, called pD3-8-12-2X, contained two copies of the chimeric methionine-rich BNP gene in tandem array.

To produce the plasmid containing four copies of the chimeric gene, the pD3-8-12-2X plasmid was digested with Eco RI and Hind III and the ends were made blunt with the Klenow fragment of DNA polymerase. A 6 kb fragment containing two copies of the chimeric gene was isolated. This fragment was ligated to the plasmid pD3-8-12-2X which had been digested with Sma I and treated with calf intestinal phosphatase. The resulting plasmid is pD3-8-12-4X.

The chimeric BNP genes were then inserted into the Ti plasmid vector pARC12. A 12 kb fragment from pD3-8-12-4X was excised by digestion with Eco RI and Hind III and ligated to pARC12 which had been digested with Eco RI and Hind III. The resulting plasmid, p12-4X, contains four copies of the BNP gene between the tDNA borders, as well as a chimeric neomycin synthase-neomycin phosphotransferase II gene for selection in plant cells. The plasmid was then transferred from *E. coli* to *Agrobacterium tumefaciens* strain LBA 4404 by triparental mating. The identities of the resulting bacteria were confirmed by southern blot analysis.

#### Preparation of *Agrobacterium tumefaciens* LBA4404/p12GUSBN17 and p12-

25

#### 4X

Overnight cultures of *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary plasmid p12GUSBN17 (DP1816, one copy BNP sequence) or p12-4X (DP1813, four copies BNP sequence), grown to log phase in Minimal A medium containing tetracycline, 1.0 mg/ml, were pooled and an optical density measurement at 550 nm was taken. Sufficient volume of the culture was placed in 15 ml conical centrifuge tubes such that upon



sedimentation between 1.0 and 2.0 x 10<sup>10</sup> cells were collected in each tube, where O.D.550 1.0 = 1.4 x 10<sup>9</sup> cells/ml. Sedimentation was by centrifugation at 6000 g for 10 minutes. After centrifugation the supernatant was decanted and the tubes were held at room temperature until inoculum was needed but not longer than one hour.

### Transformation

Inoculations were conducted in batches such that each plate of seed was treated with a newly resuspended pellet of Agrobacterium. One at a time the pellets were resuspended in 20 ml inoculation medium. Inoculation medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v. 6-benzylaminopurine (BAP), 44 mM; indolebutyric acid (IBA), 0.5 mM; acetosyringone (AS), 100 mM and was buffered to pH 5.5 with MES, 10 mM. Resuspension was by vortexing. The inoculum was then poured into a petri dish containing prepared seed and the cotyledonary nodes were macerated with a surgical blade. This was accomplished by dividing seed in half by longitudinal section through the shoot apex preserving the two whole cotyledons. The two halves of the shoot apex were then broken off their respective cotyledons by prying them away with a surgical blade. The cotyledonary node was then macerated with the surgical blade by repeated scoring along the axis of symmetry. Care was taken not to cut entirely through the explant to the abaxial side. Twenty explants were prepared in roughly five minutes and then incubated for 30 minutes at room temperature without agitation. Additional plates were prepared during this time. After 30 minutes the explants were transferred to plates of the same medium solidified with Gelrite (Merck & Co., Inc.), 0.2% w/v. explants were embedded with the adaxial side up and level with the surface of the medium and cultured at 22°C for three days under cool white fluorescent light, approximately 20 mEm<sup>2</sup>S<sup>-1</sup>.

### Culture and selection

After three days the explants were moved to liquid counterselection medium. Counterselection medium consisted of B5 salts (G5893), 3.2 gm/L;

sucrose, 2.0% w/v; BAP, 5.0 mM; IBA, 0.5 mM; vancomycin, 200 mg/ml; cefotaxime, 500 mg/ml and was buffered to pH 5.7 with MES, 3 mM. Ten explants were washed in each petri dish with constant, slow gyratory agitation at room temperature for four days. Counterselection medium was replaced  
5 four times.

The explants were then picked to agarose solidified selection medium. Selection medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0%, w/v; BAP, 5.0 mM; IBA, 0.5 mM; kanamycin sulfate, 50 mg/ml; vancomycin, 100 mg/ml; cefotaxime, 30 mg/ml; timentin, 30 mg/ml and was buffered to pH  
10 5.7 with MES, 3.0 mM. Selection medium was solidified with SeaKem agarose, 0.3% w/v. The explants were embedded in the medium, adaxial side down and cultured at 28°C with a 16 hour day length and cool white fluorescent illumination of 60-80 mEm<sup>2</sup>S<sup>-1</sup>.

After two weeks explants were again washed with liquid medium on the  
15 gyrotory shaker. This time the wash was conducted overnight in counterselection medium containing kanamycin sulfate, 50 mg/ml. The following day explants were picked to agarose solidified selection medium. Again they were embedded in the medium, adaxial side down, Culture was as before for another two week period.

20

### Regeneration

After one month on selective media transformed tissue became visible as green sectors of regenerating tissue against a background of bleached, less healthy tissue. Explants without green sectors were discarded, explants  
25 with green sectors were transferred to elongation medium. Elongation medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v; IBA, 3.3 mM; gibberellic acid, 1.7 mM; vancomycin, 100 mg/ml; cefotaxime, 30 mg/ml; and timentin, 30 mg/ml, buffered to pH 5.7 with MES, 3.0 mM. Elongation medium was solidified with gelrite, 0.2% w/v. They were embedded adaxial  
30 side up and cultured as before. Culture was continued on this medium with transfers to fresh plates every two weeks. When shoots became 0.5 cm in length they were excised at the base and placed in rooting medium in 13x100

mm test tubes. Rooting medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 15 gm/L; nicotinic acid, 20 mM; pyroglutamic acid (PGA), 900 mg/L and IBA, 10 mM. It was buff red to pH 5.7 with MES, 3.0 mM and solidified with Gelrite, 0.2% w/v. After ten days the shoots were transferred to the  
5 same medium without IBA or PGA. Shoots were rooted and held in these tubes under the same environmental conditions as before.

When a root system was well established the plantlet was transferred to sterile soil mix in plant cons (ICN Biomedicals, Inc., cat. no. 26-720 & 1-02). Temperature, photoperiod and light intensity remained the same as  
10 before. Under these conditions the regenerants became vigorous, mostly normal (though small) plants. When their root systems again became well established a corner of the plant con was cut off and the plants were gradually hardened off in an environmental chamber or greenhouse. Finally they were potted in soil mix and grown to maturity, bearing seed, in a  
15 greenhouse.

#### Growth, increase, and harvest of transgenic soybeans.

Seed from untransformed and transformed plants of the same variety (9341) was planted in the spring of 1992 and harvested in the fall of 1992 in  
20 Iowa. Each individual line was kept separate while grown in one or more 10.5 foot rows for maximum increase. This is the source of seed in Table 1 and the "JH2" source in Table 2. Lines from transformation events wherein one copy of the BNP gene was inserted are referred to as BNP1X. Lines in which four copies were inserted are designated BNP4X.

25 Most of the harvested BNP4X seed in the fall of 1992 was increased in Puerto Rico. This seed was planted by line in December, 1992 and harvested by line in March, 1993. This was source "PF2" in Table 2.

Part of the increased, harvested seed was returned for yield test and further laboratory testing. The rest was replanted by line in March, 1993 and  
30 harvested by line in June, 1993 in Puerto Rico. This was the source "PS3" in Table 2. The entire second cycle increase was about 2 acres, or a little more than 0.1A per lin .

### Trypsin inhibitor analyses

Spectrophometric analysis of trypsin and chymotrypsin inhibitor activities in soybean seeds were carried out according to the published methods of Hymowitz, *et al.* (1992) *J. Agr. Food*, 40, 2356-2363. Analyses of  
5 trypsin inhibitor activity/gram (TIU/gram) and  $\alpha$ -chymotrypsin inhibitor activity/gram (CIU/gram) are presented in Tables 1 and 2.

All samples submitted contained KTI. All samples contain the Ti-A allele. All samples contained BBI activity which was measured by two different methods: (1) trypsin inhibitor activity gel; and (2)  $\alpha$ -chymotrypsin  
10 inhibitor activity gel. (BBI is a double-headed protease inhibitor, inhibiting both trypsin and  $\alpha$ -chymotrypsin).

Table 1 shows the results of analysis of twelve soybean samples for trypsin and chymotrypsin inhibitor assay activity. All samples, except the normal controls, P9341, PR, were transformed to express higher levels of the  
15 amino acid methionine. This analysis included lines containing only one copy of the BNP gene: BX1P9341-1, BX1P9341-2, BX1P9341-3, BX1P9341-4, New 1X J2R17P23-24 and New 1X J2R17P18-20. Three replicates of 20 mg samples each representing five seeds selected at random from each of the 12 soybean samples were tested.

20 Except for two BNP1X samples, New 1X J2R17P23-24 and New 1X J2R17P18-20, all transgenic seed showed a reduction in both trypsin and  $\alpha$ -chymotrypsin inhibitor activity compared to controls. In fact,  $\alpha$ -chymotrypsin inhibitor activity was reduced tenfold in one sample of BNP4X and almost 60% in overall trypsin inhibitor activity.

25 The samples analyzed in Table 2 represent successive generations of three different BNP4X sources (PS2, PF2, JH2) of the same genetic lines. Seeds from JH2 were allowed to mature longer than seeds from designated sources PS2 and PF2.

Even more striking results are shown in Table 2. Compared to wild-  
30 type seed, the reduction in trypsin and  $\alpha$ -chymotrypsin inhibitor activity is extraordinary, especially in the samples from the Iowa plots where the seeds had a longer maturation period.

TABLE 1  
Spectrophotometric analysis of trypsin and chymotrypsin inhibitor activities in the seeds

Sample	Repl.	A247/min	(TI)A247/min	TIU/gram	A256/min	(CI)A256/min	CIU/gram
BX1P9341-1	1	0.032	0.019	3518.52	0.018	0.007	54.46
BX1P9341-1	2	0.034	0.017	3148.15	0.017	0.008	62.24
BX1P9341-1	3	0.032	0.019	3518.52	0.017	0.008	62.24
BX1P9341-2	1	0.032	0.019	3518.52	0.014	0.011	85.58
BX1P9341-2	2	0.032	0.019	3518.52	0.015	0.01	77.80
BX1P9341-2	3	0.033	0.018	3333.33	0.015	0.01	77.80
BX1P9341-3	1	0.035	0.016	2962.96	0.017	0.008	62.24
BX1P9341-3	2	0.035	0.016	2962.96	0.017	0.008	62.24
BX1P9341-3	3	0.037	0.014	2592.59	0.017	0.008	62.24
BX1P9341-4	1	0.038	0.013	2407.41	0.019	0.006	46.68
BX1P9341-4	2	0.038	0.015	2777.78	0.017	0.008	62.24
BX1P9341-4	3	0.037	0.014	2592.59	0.018	0.007	54.46
P9341, PR	1	0.027	0.024	4444.44	0.009	0.016	124.48
P9341, PR	2	0.03	0.021	3888.89	0.009	0.016	124.48
P9341, PR	3	0.028	0.023	4259.26	0.009	0.016	124.48
NEW 1X J2R17P23-24	1	0.021	0.03	5555.56	0.008	0.017	132.26
NEW 1X J2R17P23-24	2	0.022	0.029	5370.37	0.008	0.017	132.26
NEW 1X J2R17P23-24	3	0.02	0.031	5740.74	0.008	0.017	132.26
NEW 1X J2R17P18-20	1	0.021	0.03	5555.56	0.008	0.017	132.26
NEW 1X J2R17P18-20	2	0.03	0.021	3888.89	0.011	0.014	108.92
NEW 1X J2R17P18-20	3	0.027	0.024	4444.44	0.009	0.016	124.48
BX4P9341-B3	1	0.041	0.01	1851.85	0.018	0.007	21.78
BX4P9341-B3	2	0.041	0.01	1851.85	0.018	0.007	21.78
BX4P9341-B3	3	0.04	0.011	2037.04	0.018	0.007	21.78
BX4P9341-A6	1	0.04	0.011	2037.04	0.018	0.007	21.78
BX4P9341-A6	2	0.04	0.011	2037.04	0.019	0.006	18.67
BX4P9341-A4	1	0.037	0.014	2592.59	0.018	0.007	21.78
BX4P9341-A4	2	0.036	0.015	2777.78	0.018	0.007	21.78
BX4P9341-A4	3	0.036	0.015	2777.78	0.017	0.008	62.24
BX4P9341-B6	1	0.042	0.009	1686.67	0.019	0.006	18.67
BX4P9341-B6	2	0.043	0.008	1481.48	0.019	0.006	18.67
BX4P9341-B6	3	0.04	0.011	2037.04	0.02	0.005	15.56
BX4P9341-C5	1	0.042	0.009	1686.67	0.021	0.004	12.45
BX4P9341-C5	2	0.043	0.008	1481.48	0.02	0.005	15.56
BX4P9341-C5	3	0.044	0.007	1296.30	0.021	0.004	12.45

TABLE 2

Spectrophotometric analysis of trypsin (TIU) and chymotrypsin (CIU) inhibitor activities in the seeds

STOCK NO.	SOURCE	A247/MIN	(TI)A247/MIN	TIU/GRAM	A256/MIN	(CI)A256/MIN	CIU/GRAM
P9341	PF2	0.006	0.044	8148	0.004	0.018	140.04
BX4P9341-9	PF2	0.035	0.015	2778	0.017	0.005	15.56
BX4P9341A4	PF2	0.022	0.028	5185	0.011	0.011	34.23
BX4P9341A6	PF2	0.032	0.018	3333	0.017	0.005	15.56
BX4P9341A7	PF2	0.036	0.014	2593	0.015	0.007	21.78
BX4P9341B3	PF2	0.034	0.016	2963	0.013	0.009	28.01
BX4P9341B6	PF2	0.035	0.015	2778	0.016	0.006	18.67
BX4P9341C2	PF2	0.033	0.017	3148	0.016	0.006	18.67
BX4P9341C5	PF2	0.034	0.016	2963	0.013	0.009	28.01
BX4P9341C7	PF2	0.025	0.025	4630	0.012	0.01	31.12
P9341	PS3	0.006	0.044	8148	0.005	0.017	132.26
BX4P9341-9	PS3	0.033	0.017	3148	0.014	0.008	24.90
BX4P9341A4	PS3	0.026	0.024	4444	0.013	0.009	28.01
BX4P9341A6	PS3	0.029	0.021	3889	0.013	0.009	28.01
BX4P9341A7	PS3	0.032	0.018	3333	0.015	0.007	21.78
BX4P9341B3	PS3	0.031	0.019	3519	0.014	0.008	24.90
BX4P9341B6	PS3	0.036	0.014	2593	0.017	0.005	15.56
BX4P9341C2	PS3	0.034	0.016	2963	0.014	0.008	24.90
BX4P9341C5	PS3	0.031	0.019	3519	0.013	0.009	28.01
BX4P9341C7	PS3	0.032	0.018	3333	0.014	0.008	24.90
P9341	JH2	0.009	0.041	7593	0.007	0.015	116.70
BX4P9341-9	JH2	0.039	0.011	2037	0.02	0.002	6.22
BX4P9341A4	JH2	0.039	0.011	2037	0.022	0	<3.11
BX4P9341A6	JH2	0.035	0.015	2778	0.018	0.004	12.45
BX4P9341A7	JH2	0.035	0.015	2778	0.019	0.003	9.34
BX4P9341B3	JH2	0.035	0.015	2778	0.019	0.003	9.34
BX4P9341B6	JH2	0.034	0.016	2963	0.018	0.004	12.45
BX4P9341C2	JH2	0.038	0.012	2222	0.021	0.001	3.11
BX4P9341C5	JH2	0.037	0.013	2407	0.02	0.002	6.22
BX4P9341C7	JH2	0.038	0.012	2222	0.022	0	<3.11

Amino acid analysis

- Th amino acid content of seeds from transformed and untransformed plants was analyzed by methods described in the *Official Methods of Analysis of the AOAC* (1990) Hilrich, K. (ed.), AOAC International, Vol. 2, pp1096-1097. The methionine content of transformed seed is shown in Tables 3 and 4 and is considerably increased compared to the untransformed seed. The relative content of all other amino acids measured remained the same in all samples.
- 5

TABLE 3  
Amino Acid Analysis

SOURCE	ALAI	AGRI	ASPI	CYSI	GLUI	GLYI	HISI	ILEI	LEUI	LYSI	METI	PHEI	PRLI	SERI	THRI	TYRI	VALI
P6341	1.36	2.64	3.25	0.48	4.61	1.19	1.07	1.74	2.35	2.29	0.51	2.06	2.06	1.59	1.54	1.86	1.63
BX4P341-9	1.74	3.28	4.25	0.69	6.55	1.42	1.06	1.76	2.92	2.36	0.82	1.94	2.46	1.69	1.47	1.44	1.76
BX4P341A4	1.34	3.07	3.81	0.63	6.52	1.27	0.97	1.59	2.70	2.20	0.81	1.83	2.29	1.63	1.29	1.33	1.61
BX4P341A6	1.31	3.04	3.77	0.60	6.56	1.26	0.96	1.58	2.69	2.16	0.81	1.82	2.32	1.65	1.27	1.32	1.60
BX4P341A7	1.32	2.99	3.75	0.59	6.35	1.25	0.96	1.56	2.65	2.15	0.81	1.78	2.20	1.62	1.30	1.30	1.58
BX4P341B3	1.49	2.92	3.75	0.58	6.36	1.33	0.96	1.54	2.61	2.17	0.70	1.77	2.30	1.67	1.31	1.30	1.56
BX4P341B6	1.30	3.02	3.77	0.61	6.55	1.26	0.96	1.57	2.67	2.16	0.76	1.80	2.32	1.65	1.29	1.31	1.59
BX4P341C2	1.28	3.36	3.74	0.60	6.46	1.24	0.94	1.54	2.64	2.12	0.84	1.77	2.26	1.62	1.29	1.30	1.55
BX4P341C5	1.29	3.03	3.71	0.66	6.50	1.25	0.95	1.56	2.65	2.14	0.79	1.79	2.21	1.62	1.29	1.31	1.58
BX4P341C7	1.27	3.28	3.67	0.53	6.36	1.24	0.93	1.52	2.60	2.10	0.83	1.74	2.26	1.60	1.28	1.28	1.53
P3041	1.34	3.07	3.99	0.60	6.48	1.31	0.94	1.66	2.68	2.31	0.57	1.83	2.31	1.68	1.38	1.32	1.64
BX4P3041-9	1.56	3.42	4.00	0.52	6.71	1.33	0.97	1.66	2.78	2.24	0.75	1.91	2.38	1.69	1.33	1.35	1.65
BX4P3041A4	1.53	3.40	3.91	0.51	6.66	1.30	0.96	1.62	2.75	2.20	0.78	1.87	2.37	1.67	1.29	1.33	1.61
BX4P3041A6	1.51	3.51	3.88	0.45	6.75	1.29	0.95	1.62	2.75	2.17	0.79	1.87	2.44	1.65	1.28	1.32	1.60
BX4P3041A7	1.60	3.57	3.95	0.50	6.34	1.30	0.97	1.65	2.77	2.15	0.78	1.86	2.36	1.56	1.31	1.19	1.63
BX4P3041B3	1.36	3.16	3.91	0.60	6.64	1.29	0.98	1.65	2.81	2.22	0.81	1.91	2.30	1.65	1.30	1.35	1.65
BX4P3041B6	1.50	3.46	3.88	0.53	6.68	1.28	0.97	1.61	2.76	2.14	0.77	1.89	2.32	1.66	1.27	1.35	1.60
BX4P3041C2	1.49	3.46	3.86	0.53	6.63	1.28	0.96	1.61	2.74	2.15	0.72	1.88	2.36	1.63	1.27	1.33	1.60
BX4P3041C5	1.58	3.67	3.95	0.49	6.22	1.29	0.97	1.66	2.79	2.14	0.78	1.87	2.28	1.55	1.29	1.36	1.64
BX4P3041C7	1.52	3.59	3.92	0.47	6.73	1.31	0.97	1.63	2.77	2.18	0.80	1.90	2.44	1.67	1.26	1.36	1.62



TABLE 4  
Amino Acid Analysis

Variable	N	NMISS	MAX	MIN	MEAN	SUM	STD	CV
SOURCE	20	0						
ALAI	20	0	1.74	1.27	1.44	28.71	0.133	9
ARGI	20	0	3.67	2.64	3.25	64.94	0.270	8
ASPI	20	0	4.25	3.25	3.84	76.72	0.191	5
CYSI	20	0	0.69	0.45	0.56	11.16	0.066	12
GLUI	20	0	6.75	4.61	6.43	128.66	0.454	7
GLYI	20	0	1.42	1.19	1.28	25.69	0.047	4
HISI	20	0	1.07	0.93	0.97	19.40	0.035	4
ILEI	20	0	1.76	1.52	1.62	32.33	0.063	4
LEUI	20	0	2.92	2.35	2.70	54.08	0.114	4
LYSI	20	0	2.36	2.10	2.19	43.75	0.067	3
METI	20	0	0.84	0.51	0.76	15.23	0.084	11
PHEI	20	0	2.06	1.74	1.85	37.09	0.074	4
PRLI	20	0	2.46	2.06	2.31	46.24	0.092	4
SERI	20	0	1.69	1.55	1.64	32.75	0.040	2
THRI	20	0	1.54	1.26	1.32	26.31	0.071	5
TYRI	20	0	1.86	1.19	1.35	27.01	0.128	10
VALI	20	0	1.76	1.53	1.61	32.23	0.048	3

### Polypeptide synthesis during late seed development

Twelve seeds were selected from transgenic (BNP4X) plants and twelve from untransformed controls, both from full pods just prior to browning and desiccation (i.e., very late in seed development). The seeds were divided into halves wherein one cotyledon was cultured in media supplemented with methionine and the other was cultured in the same media without methionine. After six days of culture the cotyledons were washed in distilled water and lyophilized.

On a dry weight basis, methionine-fed controls gained 3% more dry weight than their counterparts cultured in media without methionine. Methionine-fed BNP cotyledons gained 34% more dry weight than their counterparts cultured in media without methionine, suggesting that late in development these beans can use methionine to produce BNP.

Coomassie blue-stained PAGE separations of the proteins showed an increase in BNP as a direct result of feeding. These gels also show that the band which is commonly assumed to be naturally-occurring, 2S seed storage protein, a methionine-rich protein that disappears when BNP is expressed in transgenic soybeans, is present when the transgenic beans are fed methionine, demonstrating that methionine source is limiting storage protein accumulation.

Following amino acid analysis, the methionine-fed BNP samples (BNP+) were found to have a 20% increase in methionine in protein compared to the BNP samples cultured without methionine (BNP-). (Table 5) The untransformed control samples incubated with methionine (Control+) had a 19% increase in methionine in protein compared to the untransformed controls incubated without methionine (Control-). (A comparison of BNP-/Control- and BNP+/Control+ demonstrates how much methionine in protein results from the synthesis of BNP. These figures suggest more than an additive effect indicating that BNP selectively pulls methionine out of the amino acid pool.)

Under methionine-limiting conditions (BNP-, Control-), the transformed seeds accumulate more methionine-containing protein than the controls. When cultured with methionine, the transformed seeds also accumulate more methionine-containing protein than the controls (BNP+, BNP-). All of these results indicate that methionine is more likely to be incorporated into BNP than other proteins such as protease inhibitors.

TABLE 5

	Sample	Met levels (weight%)
5	BNP+	1.21
	BNP-	1.01
	Control+	0.88
	Control-	0.74
10	Control+/Control-	+19%
	BNP+/BNP-	+20%
	BNP-/Control-	+36%
	BNP+/Control+	+38%

15        The cotyledons from each of the experimental groups were ground into meal, defatted, and analyzed for trypsin and chymotrypsin inhibitor activity as previously described. (Table 6). Three replicates of meal from each of the experimental groups were analyzed.

20        Transformed seeds supplemented with methionine (BNP+) demonstrate trypsin inhibitor activity levels equal to or higher than that of unsupplemented controls (Control-). The  $\alpha$ -chymotrypsin inhibitor activity level of the BNP+ meal was as high as that of the controls cultured with methionine (Control+). From these results, it is clear that methionine is limiting to the synthesis of KTI and BBI protease inhibitors and the BNP

25        protein is a sink for methionine that would have been used to synthesize these inhibitors.

TABLE 6

Spectrophotometric Analysis of Trypsin (TIU) and Chymotrypsin (CIU) Inhibitor Activities in the Defatted Seed Meal

Sample #	Replication	A247/min	(TI) A247/min	TIU/g seed meal	A256/min	(CI) A256/min	CIU/g seed meal
BNP +	A	0.025	0.02	7407	0.006	0.015	311
BNP +	B	0.023	0.022	8148	0.006	0.015	311
BNP +	C	0.023	0.022	8148	0.006	0.015	311
BNP -	A	0.037	0.008	2963	0.019	0.002	41
BNP -	B	0.036	0.009	3333	0.018	0.003	62
BNP -	C	0.036	0.01	3704	0.019	0.002	41
Control +	A	0.014	0.031	11481	0.005	0.016	332
Control +	B	0.015	0.03	11111	0.006	0.015	311
Control +	C	0.016	0.029	10741	0.006	0.015	311
Control -	A	0.023	0.022	8148	0.01	0.011	228
Control -	B	0.024	0.021	7778	0.01	0.011	228
Control -	C	0.024	0.021	7778	0.01	0.011	228

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Variations on the above embodiments are within the ability of one of ordinary skill in the art, and such variations do not depart from the scope of the present invention as described in the following claims.

We claim:

1. A plant seed that is genetically modified, relative to a wild type of the species of said seed, to preferentially express a preselected protein,  
5 whereby the content of a second, endogenous protein is diminished in the seed.
2. A plant seed according to claim 1, wherein said preselected protein is selected from the group consisting of a methionine-containing protein, a  
10 glycine-rich protein, a lysine-rich protein, a cysteine-containing protein, a tryptophan-containing protein, and a tyrosine-containing protein.
3. A plant seed according to claim 1, wherein said preselected protein is a second endogenous protein which said seed is genetically modified to  
15 overexpress.
4. A plant seed according to claim 1, wherein said preselected protein is a heterologous protein that said seed is genetically modified to express.
- 20 5. A plant seed according to claim 1, wherein said species is *Glycine max*.
6. A plant seed according to claim 5, wherein said endogenous protein is an antinutritional protein.  
25
7. A plant seed according to claim 5, wherein said endogenous protein is a protease inhibitor.
8. A method of reducing the level of an endogenous protein in a seed  
30 comprising expressing a second protein in said seed that requires the same amino acid for synthesis as the endogenous protein.